

Lipolytic Enzymes Involved in the Virulence of Human Pathogenic Fungi

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Abstract Pathogenic microbes secrete various enzymes with lipolytic activities to facilitate their survival within the host. Lipolytic enzymes include extracellular lipases and phospholipases, and several lines of evidence have suggested that these enzymes contribute to the virulence of pathogenic fungi. *Candida albicans* and *Cryptococcus neoformans* are the most commonly isolated human fungal pathogens, and several biochemical and molecular approaches have identified their extracellular lipolytic enzymes. The role of lipases and phospholipases in the virulence of *C. albicans* has been extensively studied, and these enzymes have been shown to contribute to *C. albicans* morphological transition, colonization, cytotoxicity, and penetration to the host. While not much is known about the lipases in *C. neoformans*, the roles of phospholipases in the dissemination of fungal cells in the host and in signaling pathways have been described. Lipolytic enzymes may also influence the survival of the lipophilic cutaneous pathogenic yeast *Malassezia* species within the host, and an unusually high number of lipase-coding genes may complement the lipid dependency of this fungus. This review briefly describes the current understanding of the lipolytic enzymes in major human fungal pathogens, namely *C. albicans*, *C. neoformans*, and *Malassezia* spp.

Keywords *Candida albicans*, *Cryptococcus neoformans*, Lipase, Lipolytic enzyme, *Malassezia*, Phospholipase

Pathogenic microbes secrete hydrolytic enzymes that enable them to breach and invade host tissues. The most highly recognized extracellular hydrolytic enzymes include proteinases and the lipolytic enzymes, lipases and phospholipases. Numerous studies have focused on the functions of proteinases in the virulence of pathogenic microbes and have suggested that proteinase activities alter the permeability of the epithelial barrier and induce inflammatory responses [1]. In contrast, the roles of lipases and phospholipases in virulence remain widely unexplored.

Lipases catalyze the hydrolysis of ester bonds of triacylglycerols, resulting in the release of fatty acids. In almost all organisms, lipases play essential roles in lipid metabolism, including digestion, transport, and the processing

of dietary lipids. In addition, a number of studies have shown that human microbial pathogens utilize lipases in their pathogenesis. Lipase activity is required for the colonization and persistence of the bacterial pathogens *Propionibacterium acnes* and *Staphylococcus epidermidis* on human skin [2, 3]. Furthermore, the lipases of *Staphylococcus aureus* and *Pseudomonas aeruginosa* have been shown to interfere with the immune response of host cells [4]. *Mycobacterium tuberculosis* also relies on its lipases to hydrolyze host cell lipids during infection, and then uses the released fatty acids as its long-term energy source [5]. Lipolytic enzymes have also been implicated in the virulence of fungal pathogens; the contribution of lipases in fungal pathogenesis has been extensively characterized in *Candida* spp. *C. albicans* possesses at least 10 lipase-encoding genes, the expression of which is largely influenced by the stage of infection [6]. In *C. parapsilosis*, lipases are responsible for the destruction of epidermal and epithelial tissues [7]. Lipases also play important roles in the virulence of skin-associated lipophilic fungal pathogens of the *Malassezia* spp. The gene that encodes for lipases in *M. furfur*, *M. pachydermatis*, and *M. globosa* has been identified, and recent genome sequencing efforts have revealed at least 14 lipase-encoding genes in *M. globosa* [8-10].

Phospholipases hydrolyze one or more ester linkages in glycerophospholipids, resulting in the release of free fatty acids [11]. In general, phospholipases are classified into 5 subclasses: A (PLA), A2 (PLA2), B (PLB), C (PLC), and D (PLD), depending on the specific ester bond they target [11].

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Evidence indicating that phospholipases may contribute to host cell penetration, injury, and lysis has emerged from the study of parasitic protozoa such as *Toxoplasma gondii* and *Entamoeba histolytica*, and bacterial pathogens of the *Rickettsia* spp. and *S. aureus*, and fungal pathogens *C. albicans*, *C. neoformans*, and *Malassezia* spp. [11, 12]. The type of phospholipases associated with pathogenicity varies among organisms, but the activities of these enzymes commonly result in the destabilization of the host cell membrane and the release of lipid second messengers [12, 13]. Thus, accumulating data have suggested the importance of lipases and phospholipases in virulence of pathogenic microbes. In this review, we summarize current findings on the roles of lipases and phospholipases in virulence of major fungal pathogens, *C. albicans* and *C. neoformans*, as well as the recently emerging cutaneous opportunistic pathogen, *Malassezia* spp.

LIPOLYTIC ENZYMES OF *CANDIDA ALBICANS*

C. albicans is the most prevalent cause of hospital-acquired infectious fungal diseases. For the immunocompromised individual, this fungus causes life-threatening systemic diseases, and its mortality may be as high as 50%. Superficial fungal infections such as thrush and vaginitis are also caused by *C. albicans* [14, 15]. The secreted lipase activity of *C. albicans* was first detected by Fu *et al.* [16]. A subsequent genomic library screening has identified the gene, *LIP1* that encodes a lipase containing the conserved Gly-X-Ser-X-Gly motif. Non-*albicans* *Candida* spp. such as *C. parapsilosis*, *C. tropicalis*, and *C. krusei* have also been shown to possess a gene that is orthologous to *C. albicans* *LIP1*. The discovery of *LIP1* in *C. albicans* has led to the identification of additional 9 lipase-encoding genes, *LIP2*~*LIP10*, wherein the sequence is highly conserved (up to 80% sequence identity). The contribution of the lipase to *C. albicans* persistence and virulence has also been suggested, based on the observation of expression of all 10 lipases during the yeast-hypha transition and the detection of transcription of lipases such as *LIP5*, *LIP6*, *LIP8*, and *LIP9* during experimental infections [6]. The differential expression of *LIP1*~*LIP10* during colonization in experimental models and in patient specimens has also been previously reported [17, 18]. Furthermore, an additional ~70 kDa extracellular lipase activity has been detected and shown to exert cytotoxic effects in the host macrophages and hepatocytes, presumably through the production of reactive oxygen species [19, 20].

Extracellular phospholipases also play major roles in the virulence of *C. albicans*, and a previous study has shown that approximately 79% of clinically isolated *C. albicans* strains secrete the enzyme [21]. PLA, PLB, PLC, and PLD have been detected in *C. albicans*, and the genes encoding these proteins have been identified [22-26]. *PLB1* encodes PLB and was the first phospholipase gene identified in *C. albicans*. A strain lacking *PLB1* has been generated and

used in the assessment of the virulent attributes of PLB. Although *PLB1* did not influence the growth and morphology of the fungus or its adherence to the host cell, the virulence of the null mutant was significantly attenuated in murine models of disseminated candidiasis [24, 27]. The second gene, *PLB2*, which encodes for PLB, has also been identified in *C. albicans* [28]. However, its role may be marginal, because *in vivo* analysis of 137 human subjects with oral and vaginal candidiasis revealed that only *PLB1* but not *PLB2* expression correlates with human oral infections [29]. To date, 3 genes, *PLC1*, *PLC2*, and *PLC3*, which encode PLC, have been identified in *C. albicans*. Among these, *PLC1* has been determined as essential, whereas *PLC2* and *PLC3* are not. Although the heterozygous *plc1/PLC1* mutant and the mutant lacking *PLC2* and *PLC3* were deficient in hyphal formation, the functions of PLC were shown to be dispensable for virulence [30]. PLD is involved in diverse essential cellular processes, including sporulation, growth, and membrane lipid synthesis in the non-pathogenic model yeast *Saccharomyces cerevisiae* [31-34]. Relevant roles for PLD in *C. albicans* have been previously demonstrated by McLain and Dolan [35], and the gene encoding the protein with a highly conserved PLD motif was identified and designated as *PLD1* [36]. An attenuation of virulence was observed in mice orally infected with a *PLD1*-deficient mutant, thus suggesting that PLD may play a role in the pathogenesis of *C. albicans* [26]. Accumulating data therefore indicate that lipolytic enzymes, such as lipases and phospholipases, play critical roles in virulence of *C. albicans*.

LIPOLYTIC ENZYMES OF *CRYPTOCOCCUS NEOFORMANS*

C. neoformans is an encapsulated human pathogenic fungus that causes pulmonary cryptococcosis and cryptococcal meningitis mainly in immunocompromised individuals such as acquired immunodeficiency syndrome (AIDS) patients [37, 38]. To date, little information on the functions and the roles of lipases in *C. neoformans* is available, although genome sequence data have indicated that the fungus possesses at least 3 lipase genes. Unlike *C. albicans*, none of the lipase-encoding genes in *C. neoformans* produce a protein with a signal peptide and no extracellular lipase activity has been detected from a clinically isolated *C. neoformans* strain [39]. In contrast, numerous studies have suggested that phospholipases play a role in the physiology and virulence of *C. neoformans*. Early studies have reported that clinically isolated *C. neoformans* strains secrete phospholipases, and a characterization of their activity has identified these as mainly phospholipase B and suggested that the activity of this enzyme correlates with the capsule size and virulence of the fungus [40-42]. Phospholipase A activity has also been previously reported, although the gene encoding this particular enzyme and its virulence attributes have not been established [43, 44]. The gene encoding phospholipase B, *PLB1*, has been identified and a

deletion mutant has been generated through targeted gene disruption. The *plb1* mutant displayed no particular phenotype when it was examined for the expression of major virulence factors such as the ability to grow at 37°C, capsule formation, and melanin synthesis. However, deletion of *PLB1* resulted in attenuated virulence in the mouse inhalation model and the rabbit meningitis model, indicating that PLB contributes to the pathogenicity of *C. neoformans* [13]. The importance of PLB in the virulence of *C. neoformans* was further confirmed by the observation of glycerophosphorylcholine (GPC) in lesions extracted from animals infected with *C. neoformans*. The occurrence of GPC was consistent with the generation of hydrolysis products from the host phospholipids through the action of PLB in *C. neoformans* [45]. Furthermore, the study suggested that PLB activity is required for the initiation of interstitial pulmonary infections and for the dissemination from the lung through the lymphatics and blood, whereas the dissemination of cryptococci to the central nervous system was shown to be independent of the enzyme [46]. *C. neoformans* PLB contains an N-terminal secretory leader peptide and acquires a glycosylphosphatidylinositol (GPI) anchor that facilitates its attachment to the plasma membrane prior to secretion [47]. PLB in *C. neoformans* is also extensively glycosylated at its N-terminal, which influences membrane association and the secretion of the enzyme [48]. Chayakulkeeree *et al.* [49] demonstrated that cleavage of PLB is mediated by phosphatidylinositol-specific phospholipase C (PI-PLC), which is encoded by two genes, *PLC1* and *PLC2*. The mutant that lacks either *PLC1* or *PLC2* was constructed to characterize the functions of PI-PLC in *C. neoformans*. While no particular phenotypic characteristic was observed in the *plc2* mutant, the effect of the deletion of *PLC1* was dramatic. The *plc1* mutant showed an impaired growth at 37°C, protein secretion, and melanin production, and was avirulent in a murine inhalation model of cryptococcosis. A correlation between PLC and the protein kinase C/mitogen-activated protein kinase signaling pathway has also been suggested and has further confirmed the significant roles of PLC in the physiology and pathogenicity of *C. neoformans*.

LIPOLYTIC ENZYMES OF MALASSEZIA SPP.

Malassezia spp. are associated with various dermatological diseases, including seborrheic dermatitis, dandruff, atopic dermatitis, and pityriasis versicolor [50]. To date, 14 spp. have been identified and all *Malassezia* spp., except *M. pachydermatis*, are known to be obligatorily lipid-dependent [51]. The lipophilic nature of *Malassezia* spp. may be attributable to its inability to synthesize myristic acid, which is the precursor of long chain fatty acids. Recent genome analysis of *M. globosa* has revealed the absence of a gene that influences fatty acid synthesis [52]. The genome of *M. globosa* contains a number of genes that encode for lipolytic enzymes, including 14 lipases and 9 phospholipases, which

may compensate for the lipid dependency of the fungus. A significantly higher lipid content in the cell wall of *Malassezia* spp. compared to non-pathogenic fungi such as *S. cerevisiae* has been previously reported, and the lipid microfibrillar layer of the *Malassezia* cell has been shown to down-regulate the inflammatory immune response, implying that the lipid dependency of the fungus might play a role in virulence [53, 54].

The importance of the lipase function was first studied in *M. furfur*. Its enzyme activity was initially detected in the insoluble fraction of the cells, and its optimum pH was determined as acidic. A positive correlation between lipase activity and cell growth has also been observed [55]. Brunke and Hube [56] cloned and characterized the gene, *MfLIP1*, which encodes for the extracellular lipase in *M. furfur*. A protein sequence analysis has shown that MfLip1 contains the conserved lipase motif, Gly-X-Ser-X-Gly, and is similar to lipases in *C. albicans*. The protein contains a signal peptide at its N-terminus, but not a transmembrane domain or a GPI-anchor motif. The *MfLIP1* gene might not belong to other lipase gene families in the genome of *M. furfur*, because no signal was detected during Southern blot analysis by using the cDNA fragment of the gene as a probe. Interestingly however, the same approach using the genome of *M. pachydermatis* displayed signals, thus suggesting the presence of an ortholog. Moreover, heterologous expression of the cDNA of *MfLIP1* in *Pichia pastoris* has suggested that the recombinant MfLip1 is most active at 40°C and its optimal pH was 5.8.

The gene encoding for the extracellular lipase in *M. pachydermatis* has been cloned and characterized [57]. *M. globosa*, one of the most frequently isolated *Malassezia* spp. from patients with dandruff and seborrheic dermatitis, also displayed extracellular lipase activity. Although recent genome analysis has revealed there are at least 14 lipase-encoding genes, only one gene, *MgLIP1*, has been identified. Similar to lipases in other *Malassezia* spp., the protein product of *MgLIP1* contains the conserved lipase motif and the signal peptide. The regulation of gene expression has also been investigated, with *MgLIP1* transcription levels regulated by its growth phase *in vitro*. The expression of *MgLIP1* was higher in the late log phase compared to early log and stationary phases. Moreover, the expression of *MgLIP1* was detected in human scalp specimens, indicating that lipases may play a key role in virulence of *M. globosa* [8]. The structure of MgLip1 has been proposed, and its substrates have been reported to be strictly specific for mono- and diacylglycerol, but not triacylglycerol [58, 59]. Other than MgLip1, additional extracellular lipase activity has been identified. The enzyme has been designated as MgLip2 and optimal activity has been observed at 30°C and pH 5.0 [60]. Interestingly, a recent study compared the extracellular lipase activity of several *Malassezia* spp., with *M. globosa* showing the highest lipase activity [61].

Current information on phospholipases in *Malassezia* spp. is limited. Juntachai *et al.* [61] detected extracellular

Table 1. Genes encoding lipases and phospholipases in major human fungal pathogens

Fungal pathogen	Lipase	Phospholipase	Virulence of the null mutant	Reference
<i>Candida albicans</i>	<i>LIP1-10</i>		N/A ^a	[16-18]
		<i>PLB1</i>	Attenuated	[27]
		<i>PLB2</i>	N/A	[28]
		<i>PLC1, PLC2, PLC3</i>	Virulent ^b	[30]
		<i>PLD1</i>	Attenuated	[26]
<i>Cryptococcus neoformans</i>	N/A			
		<i>PLB1</i>	Attenuated	[13]
		<i>PLC1</i>	Avirulent	[49]
<i>Malassezia</i> spp.	<i>MfLIP1</i> <i>MpLIP1</i> <i>MgLIP1</i> <i>MgLIP2</i>	<i>PLC2</i>	Virulent	
			N/A	[56]
			N/A	[57]
			N/A	[8]
		N/A	[60]	
		N/A		

N/A, not available.

^aGenes have been identified, but the function and virulence attributes have not been established.

^bThe null mutant was as virulent as the wild-type in the experimental animal model.

phospholipase activities in *M. furfur*, *M. pachydermatis*, *M. slooffiae*, *M. sympodialis*, *M. globosa*, *M. restricta*, and *M. obtusa*. However, enzyme activity was higher only in *M. pachydermatis*, which is lipid-independent and has been normally isolated in dogs. The phospholipase activity in *Malassezia* spp. associated with humans is relatively low, which implies that the role of secreted phospholipases in *Malassezia* spp. might be marginal.

CONCLUSIONS

Numerous experimental data have revealed the contribution of lipolytic enzymes in the virulence of human pathogenic fungi (Table 1). *C. albicans* and *C. neoformans* have served as good model systems for investigating the functions and roles of specific enzymes in fungal pathogenesis. Lipolytic enzymes, which include secreted lipases and phospholipases, have been shown to influence growth, morphology, adherence, and dissemination of fungal cells across the host. Several studies have also emphasized the significance of lipolytic enzymes in *Malassezia* spp., although no genetic tool has been developed. Secreted lipases are key players and may complement lipid dependency of *Malassezia* spp. Therefore, a greater effort in understanding the roles of lipolytic enzymes in fungal pathogens is warranted.

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